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# Natural sweetening of food products by engineering *Lactococcus lactis* for glucose production

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## Abstract

We show that sweetening of food products by natural fermentation can be achieved by a combined metabolic engineering and transcriptome analysis approach. A *Lactococcus lactis* ssp. *cremoris* strain was constructed in which glucose metabolism was completely disrupted by deletion of the genes coding for glucokinase (*glk*), EII<sup>man/glc</sup> (*ptmABCD*), and the newly discovered glucose-PTS EII<sup>cel</sup> (*ptcBAC*). After introducing the lactose metabolic genes, the deletion strain could solely ferment the galactose moiety of lactose, while the glucose moiety accumulated extracellularly. Additionally, less lactose remained in the medium after fermentation. The resulting strain can be used for in situ production of glucose, circumventing the need to add sweeteners as additional ingredients to dairy products. Moreover, the enhanced removal of lactose achieved by this strain could be very useful in the manufacture of products for lactose intolerant individuals.

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**Keywords:** *Lactococcus lactis*; Glucose; Lactose; Natural sweetening

## 1. Introduction

Nutraceuticals comprise a wide range of foods or food components, including fermenting bacteria, with a claimed medical or health benefit. *Lactococcus lactis* is a lactic acid bacterium used in the dairy industry for the production of fermented milk products and, thus, is a good target for the production of nutraceuticals. Additionally, *L. lactis* is a suitable model organism for metabolic pathway engineering (Kleerebezem and Hugenholtz, 2003), since it has a relatively simple carbon metabolism and many molecular cloning tools are available (Kuipers et al., 1998; Leenhouts et al., 1996, 1998). The metabolism of *L. lactis* has already been successfully engineered, e.g., for the production of the sweet amino acid L-alanine (Hols et al., 1999), the production of the buttery flavor diacetyl (Hugenholtz et al., 2000), the production of mannitol (Gaspar et al.,

2004; Wisselink et al., 2005), and for the simultaneous overproduction of the vitamins folate and riboflavin (Sybesma et al., 2004). The aim of the present study was to disrupt glucose uptake and metabolism in *L. lactis* in such a way that, when growing on lactose it excretes glucose, which can be used as a natural sweetener in dairy products. A second goal was to reduce lactose contents in the final product.

Like in many other bacteria, the phosphoenolpyruvate:sugar phosphotransferase system (PEP:PTS), mediating uptake and phosphorylation of carbohydrates (Postma et al., 1993), (Saier et al., 1996), is the main sugar transport system in *L. lactis*. Uptake of glucose in *L. lactis* can take place either via these PEP:PTS systems or via one or more non-PTS transporter(s), after which the sugar is phosphorylated to glucose-6-phosphate by glucokinase. Subsequently, glucose-6-phosphate enters glycolysis (Fig. 1). PEP:PTS systems for six different (types of) sugars, i.e., fructose, mannose, sucrose, mannitol,  $\beta$ -glucosides and cellobiose, have been annotated in the nucleotide sequence

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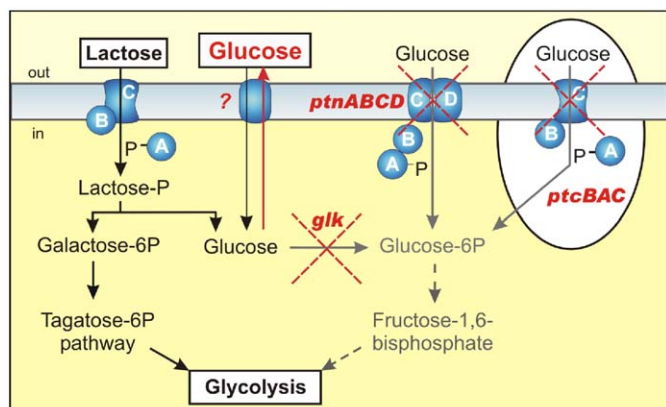


Fig. 1. Model of glucose metabolism in *L. lactis* NZ9000. Lactose is taken up by the lactose-PTS specified by the resident plasmid pMG820 (Lac<sup>+</sup>). The galactose moiety of lactose is used for metabolism via the tagatose-6-phosphate pathway, also supplied in pMG820. The glucose moiety is phosphorylated by glucokinase before entering glycolysis. Glucose can be imported via either the PTS<sup>man/glc</sup> (*ptnABCD*) or the newly discovered PTS<sup>cel</sup> (*ptcBAC*, encircled), consisting of a cytosolic EIIAB-part and an integral membrane EIIC-part or a cytosolic EIIAB-part and an integral membrane EIIC-part, respectively. Dashed crosses: *glk*, *ptnABCD*, and *ptcBA* deletions made in this study. Red arrow: excretion of glucose as observed in *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup>. Question-mark: unidentified glucose transporter(s).

of the genome of *L. lactis* ssp. *lactis* IL1403 (Bolotin et al., 2001). Homologues of these PTS genes are present in the genome sequence of *L. lactis* ssp. *cremoris* MG1363 (Zomer et al., personal communication on behalf of RUG, UCC, IFR MG1363 sequence consortium). The mannose/glucose-PTS is considered to be the main uptake system for glucose (Thompson et al., 1985). The milk-sugar lactose is imported by a dedicated PEP:PTS<sup>lac</sup>. The lactose-phosphate formed during transport is hydrolyzed intracellularly to galactose-6-phosphate, which is metabolized further through the tagatose-6-phosphate pathway, and to glucose, which enters glycolysis after phosphorylation by glucokinase. The genes for lactose usage are located on the lactococcal plasmid pMG820 (Maeda and Gasson, 1986) (Fig. 1).

Directed engineering of *L. lactis* results in stable and clear genotypes, in contrast to genotypes resulting from classical mutagenesis techniques, by which various genetic changes may go undetected (Bailey, 1991; Nielsen, 2001). The directed engineering approach proposed here, involving deletion of a known and a newly discovered PTS-system specific for glucose and the gene encoding glucokinase, as well as the introduction of the lactose metabolic genes, resulted in a strain producing glucose (Fig. 1). The fate of carbon in each sugar moiety during lactose fermentation in the engineered strains was investigated using <sup>13</sup>C-labelled substrates and in vivo Nuclear Magnetic Resonance (NMR) (Neves et al., 1999). The production of glucose from lactose could be demonstrated in non-growing cells as well as during cell growth in defined medium and in skim milk.

## 2. Materials and methods

### 2.1. Microbial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. The strains were grown in M17 (Difco<sup>TM</sup> & BBL<sup>TM</sup>, Sparks, Maryland) with 0.5% galactose (w/v) or 2% lactose (w/v) at 30 °C or 37 °C, in chemically defined medium (CDM; Poolman and Konings, 1988), with 1% glucose (w/v) or 2% lactose (w/v) or in 10% reconstituted skim milk (Oxoid Ltd., Basingstoke, England). The optical density of milk-grown culture was measured using the milk clearing method of Kanasaki (Kanasaki et al., 1975), in which 100 µl of culture was mixed with 900 µl 0.5 M borate (pH 8.0) containing 10 mM EDTA; after 30 min incubation at room temperature the optical density at 600 nm was measured. When necessary, erythromycin and chloramphenicol were used at a final concentration of 5 µg/ml. For growth in a 2 L fermentor (New Brunswick BioFlo, Edison, NJ), the medium was gassed with argon for 10 min prior to inoculation (4% inoculum from a culture grown overnight); the pH was kept at 6.5 by automated addition of 5 N NaOH, and an agitation rate of 70 rpm was used. Growth was monitored by measuring the optical density at 595 or 600 nm.

### 2.2. DNA techniques

General DNA techniques were performed essentially as described (Sambrook et al., 1989). Plasmid DNA was isolated by the method of Birnboim and Doly (Birnboim and Doly, 1979). Restriction enzymes, T4 DNA ligase, Expand polymerase and Taq polymerase were obtained from Roche Applied Science (Mannheim, Germany) and used according to the supplier's instructions. PCR was performed in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany).

### 2.3. Specific cloning procedures

Gene deletions were all performed in *L. lactis* strain NZ9000 and were constructed with the help of *L. lactis* strains LL108 and LL302 and pORI280-derivatives using a two-step homologous recombination method described before (Leenhouts et al., 1998). This method does not leave antibiotic resistance markers in the chromosome, and multiple deletions in one strain can be easily realized. Primers used for cloning are listed in Table 1. Chromosomal DNA of *L. lactis* NZ9000 was used as a template in PCR amplifications. An *L. lactis* NZ9000Δ*glk* strain, in which only the first 360 bp were left of the 969 bp of *glk*, was engineered using the primers *glk5*, *glk6*, *glk7*, and *glk8*. *L. lactis* NZ9000Δ*ptnABCD*, in which *ptnAB* is disrupted after 578 bp, *ptcC* is completely deleted and the first 441 bp of *ptcD* are missing, was made using the primers *ptn1*, *ptn2*, *ptn3*, and *ptn4*. *L. lactis* NZ9000Δ*ptcBA*, carrying

Table 1  
Lactococcal strains, plasmids, and primers used

	Description	References
<i>Strain</i>		
NZ9000	Derivative of MG1363 carrying <i>pepN::nisRK</i>	Kuipers et al. (1998)
LL302	RepA + MG1363, carrying a single copy of pWV01 <i>repA</i> in <i>pepX</i>	Leenhouts et al. (1998)
LL108	RepA + MG1363, Cm <sup>r</sup> , carrying multiple copies of pWV01 <i>repA</i> in the chromosome	Leenhouts et al. (1998)
NZ9000Δ <i>glk</i>	Derivative of NZ9000 containing a 404-bp deletion in <i>glk</i>	This work
NZ9000Δ <i>ptmABCD</i>	Derivative of NZ9000 containing a 1736-bp deletion in <i>ptmABCD</i>	This work
NZ9000Δ <i>glkΔptmABCD</i>	Derivative of NZ9000Δ <i>glk</i> containing a 1736-bp deletion in <i>ptmABCD</i>	This work
NZ9000Δ <i>glkΔptcBA</i>	Derivative of NZ9000Δ <i>glk</i> containing a 657-bp deletion in <i>ptcBA</i>	This work
NZ9000Glc <sup>-</sup>	Derivative of NZ9000Δ <i>glkΔptmABCD</i> containing a 657-bp deletion in <i>ptcBA</i>	This work
NZ9000Lac <sup>+</sup>	NZ9000 carrying pMG820	This work
NZ9000Glc <sup>-</sup> Lac <sup>+</sup>	NZ9000Glc <sup>-</sup> carrying pMG820	This work
<i>Plasmid</i>		
pMG820	Lactose mini-plasmid of 23.7 kb, containing <i>lacFEGABCD</i> , derivative of pLP712	Maeda and Gasson (1986)
pORI280	Em <sup>r</sup> , LacZ <sup>+</sup> , ori <sup>+</sup> of pWV01, replicates only in strains providing RepA <i>in trans</i>	Leenhouts et al. (1996)
pORI280- <i>glk</i> <sup>r</sup>	Em <sup>r</sup> , derivative of pORI280 specific for integration in the <i>L. lactis glk</i> gene	This work
pORI280- <i>ptmABCD</i> <sup>r</sup>	Em <sup>r</sup> , derivative of pORI280 specific for integration in the <i>L. lactis ptmABCD</i> genes	This work
pORI280- <i>ptcBA</i> <sup>r</sup>	Em <sup>r</sup> , derivative of pORI280 specific for integration in the <i>L. lactis ptcBA</i> genes	This work
pVE6007	Cm <sup>r</sup> , temperature-sensitive derivative of pWV01	Maguin et al. (1992)
<i>Primer</i>	Sequence from 5' to 3'	Restriction-site
Glk5	GCTCTAGACCAGATCGTTTGGATGCG	<i>Xba</i> I
Glk6	CGCGGATCCTTAAGCAGCAACGTTAGCG	<i>Bam</i> HI
Glk7	CGCGGATCCGGTCTACTTTAAACCCCTGC	<i>Bam</i> HI
Glk8	GGAAGATCTGGATAGAAAGATTCCATCC	<i>Bgl</i> II
Ptn1	GCTCTAGAGGAGGTTATCAGATTGAG	<i>Xba</i> I
Ptn2	CCATCGATGCTCTTGAGTCTGGAGTCC	<i>Clal</i> I
Ptn3	CCATCGATCTCCACTCTCTCTTCATCG	<i>Clal</i> I
Ptn4	GAAGATCTTGAGCCACAGATTCTCTCC	<i>Bgl</i> II
Ptc1	GCTCTAGAGTCATCTCTGACCCCTTC	<i>Xba</i> I
Ptc2	CGGATCCTTAGGCTGCACATGCAAGTGC	<i>Bam</i> HI
Ptc3	CGGATCCCCCTTGCAGTAGAAGTTGTTG	<i>Bam</i> HI
Ptc4	CGGAATCCGGATAAGTTACATCGCTAAATG	<i>Eco</i> RI
	Location annealing part on chromosome	
	196–178 bp upstream of <i>glk</i> translational start site (TSS)	
	345–360 bp downstream of <i>glk</i> TSS	
	765–785 bp downstream of <i>glk</i> TSS	
	399–417 bp downstream of <i>glk</i> stop codon	
	14 bp up to 5 bp downstream of <i>ptmAB</i> TSS	
	560–578 bp downstream of <i>ptmAB</i> TSS	
	422–442 bp downstream of <i>ptmD</i> TSS	
	132–150 bp downstream of <i>ptmD</i> stop codon	
	560–541 bp upstream of <i>ptcB</i> TSS	
	19–36 bp downstream of <i>ptcB</i> TSS	
	294–313 bp downstream of <i>ptcA</i> TSS	
	509–531 bp downstream of <i>ptcA</i> stop codon	

only the first 36 bp of *ptcB* and the last 58 bp of *ptcA*, was made using the primers ptc1, ptc2, ptc3, and ptc4.

#### 2.4. Glucokinase enzymatic assays

Cells ( $\sim 10^9$ ) were harvested at mid-exponential growth-phase, resuspended in 1 ml 10 mM potassium phosphate ( $\text{KPi}$ ) buffer (pH 7.2) and disrupted with 0.5 g glass beads ( $\varnothing$  50–105  $\mu\text{m}$ , Fischer Scientific BV, Den Bosch, the Netherlands), using a Mini-BeadBeater-8 (Biospec Products, Inc., Bartlesville, OK) with two 1 min pulses of homogenization, and a 1 min interval on ice. Cell debris was pelleted and glucokinase activity in the cell-free extract was assayed spectrophotometrically by the glucose-6-phosphate dehydrogenase (Glc-6P-DH) (EC1.1.1.49): NADPH-coupled assay (Porter et al., 1982). The assay mixture contained 10 mM  $\text{KPi}$  (pH 7.2), 5 mM  $\text{MgCl}_2$ , 1 mM  $\text{NADP}^+$ , 1 mM ATP, 1 U Glc-6P-DH, 20 mM glucose and cell-free extract (usually 20  $\mu\text{l}$ ) in a total volume of 250  $\mu\text{l}$ . Protein was determined by the method of Bradford (1976).

#### 2.5. In vivo NMR

Cells were grown in medium containing 2% lactose (w/v), harvested in the mid-logarithmic phase of growth, and were resuspended in 50 mM  $\text{KPi}$  buffer (pH 6.5) to a protein concentration of approximately 15 mg protein/ml. In vivo NMR experiments were performed using the on-line system described earlier (Neves et al., 1999). Lactose specifically labeled on the galactose moiety ( $[1-^{13}\text{C}_{\text{Gal}}]\text{lactose}$ , 20 mM) or on the glucose moiety ( $[1-^{13}\text{C}_{\text{Glc}}]\text{lactose}$ , 20 mM) was added to the cell suspension at time-point zero. The time course of lactose consumption, product formation, and changes in the pools of intracellular metabolites were monitored in vivo. When the substrate was exhausted and no changes in the resonances of intracellular metabolites were observed, an NMR-sample extract was prepared as described previously (Neves et al., 1999; Neves et al., 2002). Carbon-13 spectra were acquired at 125.77 MHz on a Bruker DRX500 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). All in vivo experiments were run using a quadruple nuclei probe head at 30  $^{\circ}\text{C}$ , as described before (Neves et al., 1999). Lactate was quantified in the NMR-sample extract by  $^1\text{H}$ -NMR in a Bruker AMX300 (Bruker BioSpin GmbH). The concentration of other metabolites (e.g., glucose) was determined in fully relaxed  $^{13}\text{C}$  spectra of the NMR-sample extracts as described (Neves et al., 2002).

#### 2.6. Transcriptome analysis

mRNA-levels of *L. lactis* strains NZ9000 and NZ9000 $\Delta\text{glk}\Delta\text{ptnABCD}$  were compared by transcriptome analysis using *L. lactis* DNA-microarrays (Kuipers et al., 2002). The strains were grown independently 3 times with Cy3 and Cy5-dye-swaps of each repetition. Thus, a total of

6 hybridized slides, all containing duplicate spots of each amplicon, resulted in a maximum of 12 measurements for each gene. The experiments were performed essentially as described (van Hijum et al., 2005), with the following modifications.

**RNA-isolation.** Macaloid was prepared by resuspending 2 g Macaloid (Bentone MA, Elementis Specialities Inc, Hightstown, NJ) in 100 ml 10 mM Tris/1 mM EDTA-buffer ( $\text{T}_{10}\text{E}_1$ ) (pH 8.0), boiling for 5 min, cooling to room temperature, sonicating by burst until the macaloid formed a gel, centrifuging and resuspending in 50 ml  $\text{T}_{10}\text{E}_1$  (pH 8.0). Cells (40 ml;  $\sim 2 \times 10^9$ ) were harvested at the mid-exponential growth phase by centrifugation (13,000g, 1 min, 20  $^{\circ}\text{C}$ ) and the cell pellets were immediately frozen in liquid nitrogen. After thawing on ice, the cells were resuspended in 500  $\mu\text{l}$   $\text{T}_{10}\text{E}_1$  (pH 8.0) (treated with DEPC; Sigma-Aldrich, St. Louis, MO), and placed in a rubber sealed screw-capped tube. After adding 500 mg glass beads ( $\varnothing$  50–105  $\mu\text{m}$ ), 50  $\mu\text{l}$  10% SDS, 500  $\mu\text{l}$  phenol/chloroform, and 175  $\mu\text{l}$  Macaloid, the cells were disrupted in a Mini-BeadBeater-8 (Biospec Products, Inc.) using two 1 min pulses (homogenize), with a 1 min interval on ice. After centrifugation (20,000g, 10 min, 4  $^{\circ}\text{C}$ ), 500  $\mu\text{l}$  of supernatant was extracted with 500  $\mu\text{l}$  phenol/chloroform, centrifuged as before, followed by an extraction with only 500  $\mu\text{l}$  chloroform. After centrifugation (20,000g, 5 min, 4  $^{\circ}\text{C}$ ), total RNA was isolated from the water phase using the ‘High Pure RNA Isolation Kit’ of Roche Applied Science, according to the manufacturer’s instructions, except that final elution was performed with 50  $\mu\text{l}$  elution buffer. RNA yield was determined spectrophotometrically at 260 nm. RNA quality was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands).

**cDNA labeling and hybridization.** Single-strand reverse transcription and indirect labeling of 20  $\mu\text{g}$  of total RNA with Cy3-dCTP or Cy5-dCTP were performed with the CyScribe Post Labelling Kit of Amersham (Roosendaal, the Netherlands) according to the manufacturer’s instructions. Hybridization of the purified labeled cDNA to an aldehyde-coated glass slide (Cel Associates/Telechem International Inc., Sunnyvale, CA) on which 2108 amplicons of *L. lactis* strain IL1403 had been spotted in duplicate, was performed as described (Kuipers et al., 2002) at 42  $^{\circ}\text{C}$ .

**Bioinformatic analysis.** Spot quantitations were processed and normalized using automated grid-based Lowess transformation ( $f = 0.5$ ) software (van Hijum et al., 2003). Differentially expressed genes were selected at a *P*-value lower than 0.00001 and a ratio of 2 and higher by using a variant of the paired *t*-test on normalized ratio data (Long et al., 2001).

#### 2.7. Sugar determinations by HPLC

Samples of *L. lactis* NZ9000Glc $^-$ Lac $^+$  or *L. lactis* NZ9000Lac $^+$  grown in CDM containing 2% (w/v) lactose



were taken at different stages of growth and centrifuged (2000g, 5 min, 4 °C). Samples of milk-grown cultures of *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> or *L. lactis* NZ9000Lac<sup>+</sup> were taken at different stages of growth and cleared using the method of Kanasaki (Kanasaki et al., 1975) before centrifugation (2000g, 5 min, 4 °C). Supernatants were filtered over 0.45 µm nylon membranes (Millipore, Bedford, MA) and stored at -20 °C until analysis by high performance liquid chromatography using a refractive index detector (Shodex RI-101, Showa Denko K.K., Japan). Lactose, glucose and galactose in the supernatants of CDM-grown cultures were quantified using an Aminex HPX-87P column (Bio-Rad Laboratories Inc., Hercules, CA) at 80 °C, with H<sub>2</sub>O as the elution fluid and a flow rate of 0.6 ml/min. Lactose, glucose, and galactose in the supernatants of milk-grown cultures were quantified using an HPX-87H anion exchange column (Bio-Rad Laboratories Inc.) at 60 °C, with 5 mM H<sub>2</sub>SO<sub>4</sub> as the elution fluid and a flow rate of 0.5 ml/min.

### 3. Results

#### 3.1. Deletion of *glk* and *ptnABCD* is not sufficient to fully block glucose metabolism

As a first step in the process of blocking glucose metabolism, a deletion was made in the glucokinase gene (*glk*) in *L. lactis* ssp. *cremoris* strain NZ9000 (Fig. 1). NZ9000 showed glucokinase activity ( $0.14 \pm 0.02$  U/mg), while no glucose-phosphorylating activity was detected in NZ9000Δ*glk*. The latter strain was still able to grow in chemically defined medium (CDM) with 1% (w/v) glucose, although the growth characteristics were different from those of strain NZ9000 (Fig. 2). NZ9000Δ*glk* showed a lower maximum growth rate and the culture reached a

higher final cell density. As NZ9000Δ*glk* is unable to use intracellular glucose, or glucose imported via a non-PTS permease system, growth of this strain on glucose is strictly dependent on uptake via one or more PEP:PTS. Removal via double cross-over recombination of the genes encoding the mannose/glucose-PTS (*ptnABCD*) in *L. lactis* NZ9000Δ*glk* resulted in a strain (NZ9000Δ*glk*Δ*ptnABCD*) still able to grow on glucose (Fig. 2). Surprisingly, the growth characteristics of *L. lactis* NZ9000Δ*glk*Δ*ptnABCD* resembled those of NZ9000Δ*glk* in CDM with 1% (w/v) glucose without pH-control, although the maximum growth rate of the double mutant was slightly higher. These results indicated that, apart from EII<sup>man/glc</sup>, another efficient glucose PTS is functional in *L. lactis* NZ9000.

#### 3.2. Transcriptome analysis indicates the additional glucose PTS in *L. lactis*

To elucidate which PEP:PTS besides EII<sup>man/glc</sup> is able to transport glucose in *L. lactis* NZ9000, mRNA-levels in strains NZ9000 and NZ9000Δ*glk*Δ*ptnABCD* grown in CDM with 1% (w/v) glucose were compared using DNA-microarrays. The assumption was that the gene(s) encoding the glucose-PTS operative in NZ9000Δ*glk*Δ*ptnABCD* would be expressed at a higher level in this mutant than in *L. lactis* NZ9000. In NZ9000Δ*glk*Δ*ptnABCD*, the genes *ptcB* and *ptcA* were both overexpressed more than 5 times, while none of the other PTS-genes were significantly overexpressed (Table 2). The sequences of *L. lactis* ssp. *cremoris* MG1363 *ptcB* and *ptcA* are resp. 93% and 91% identical to the corresponding genes of *L. lactis* spp. *lactis* IL1403. *L. lactis* spp. *lactis* IL1403 and *L. lactis* spp. *cremoris* MG1363 share >85% sequence homology, enabling efficient hybridization of probes (IL1403 amplicons on the slides) and target cDNA (from MG1363), as shown previously (den Hengst et al., 2005). The genes *ptcB* and *ptcA* have been annotated as part of a cellobiose-PTS, encoding the protein complex EIIBA<sup>cel</sup> (Bolotin et al., 2001). The chromosomal organisation of the genes

Table 2

Comparison of PTS-gene expression in *L. lactis* NZ9000Δ*glk*Δ*ptnABCD* and *L. lactis* NZ9000

Gene	Annotation	Ratio <sup>a</sup>	P-value
<i>ptcB</i>	Cellobiose EIIB	5.2	3.E-08
<i>ptcA</i>	Cellobiose EIIA	5.0	7.E-07
<i>ptcC</i>	Cellobiose EIIC	1.1	8.E-02
<i>celB</i>	Cellobiose EIIC	0.8	4.E-01
<i>fruA</i>	Fructose EIIBC	0.9	3.E-01
<i>mtlD</i>	Mannitol EIIBC	1.1	7.E-01
<i>mtlF</i>	Mannitol EIIA	0.9	6.E-01
<i>ptbA</i>	β-glucoside EIABC	0.8	1.E-02
<i>yleD</i>	Sucrose EIIBC	0.9	5.E-01
<i>yleE</i>	β-glucoside EIABC	1.1	5.E-01
<i>yidB</i>	Cellobiose EIIC	1.1	1.E-01
<i>yedF</i>	β-glucoside IIABC	1.0	8.E-01

<sup>a</sup> *L. lactis* NZ9000Δ*glk*Δ*ptnABCD* over *L. lactis* NZ9000.

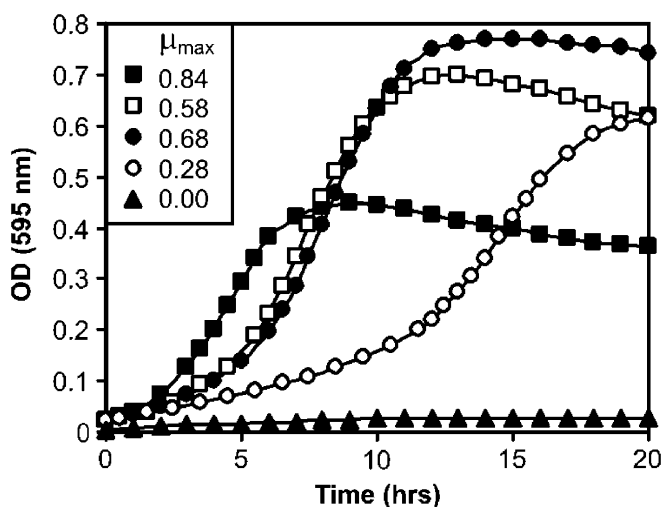


Fig. 2. Growth of *L. lactis* in glucose-containing CDM. *L. lactis* strains NZ9000 (■), NZ9000Δ*glk* (□), NZ9000Δ*glk*Δ*ptnABCD* (●), NZ9000Δ*glk*Δ*ptcBA* (○) and NZ9000Glc<sup>-</sup> (▲) were grown in CDM with 1.0% glucose at 30 °C in microtiterplates. OD<sub>595</sub> was measured at 1 hr intervals. For each strain the  $\mu_{\max}$  is presented in the inset.

encoding PTS<sup>cel</sup> is *ptcB-ptcA-yecA-ptcC*, in which *yecA* encodes a putative transcriptional regulator and *ptcC* encodes EIIC<sup>cel</sup> (Bolotin et al., 2001). To examine whether EIIC<sup>cel</sup> could use glucose as a substrate, a food-grade strain was constructed in which the *glk*, *ptnABCD*, and *ptcBA* genes were deleted. *L. lactis* NZ9000 $\Delta$ *glk* $\Delta$ *ptnABCD* $\Delta$ *ptcBA* was isolated on a medium with galactose as the sole carbon and energy source. The strain could not grow in a medium containing glucose as the sole carbon source (Fig. 2), showing that *ptcBA* is part of a glucose transporter. Removal of *glk*, *ptnABCD*, and *ptcBA* was sufficient to fully block glucose metabolism in *L. lactis* NZ9000, and this glucose-negative NZ9000-derivative will be named NZ9000Glc<sup>−</sup> from here onwards. As *L. lactis* strains NZ9000 $\Delta$ *glk* $\Delta$ *ptcBA* and NZ9000 $\Delta$ *glk* $\Delta$ *ptnABCD* could metabolize glucose (Fig. 2), both *L. lactis* EIIC<sup>cel</sup> and EIIC<sup>man/glc</sup> can use glucose as a substrate. Furthermore, the ability to use cellobiose as a substrate was heavily impaired in strain NZ9000 $\Delta$ *ptcBA* (data not shown), showing that EIIC<sup>cel</sup> also plays a role in cellobiose uptake in *L. lactis*.

### 3.3. NZ9000Glc<sup>−</sup>Lac<sup>+</sup> produces glucose under several conditions

Lactococcal plasmid pMG820, carrying the genes for lactose-PTS and the tagatose-6-phosphate pathway (Maeda and Gasson, 1986), was introduced in *L. lactis* strains NZ9000 and NZ9000Glc<sup>−</sup>, providing both strains with the ability to use lactose as a substrate (Lac<sup>+</sup>). Growth of the two resulting strains in CDM with 2% (w/v) lactose was analyzed in batch cultures with and without pH control. Sugar (lactose, glucose, and galactose) concentrations in the medium were determined at several points during growth using high performance liquid chromatography (HPLC). *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> had a lower maximum growth rate and the culture-pH dropped more slowly than that of *L. lactis* NZ9000Lac<sup>+</sup> when the strains were grown as standing cultures without pH-control. (Fig. 3a and b). Under these conditions *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> used more lactose from the medium (the concentration was lowered by 16.7 mM, compared to 8.0 mM by NZ9000Lac<sup>+</sup>) before growth ceased by the lowered pH. Unlike *L. lactis* NZ9000Lac<sup>+</sup>, which did not produce glucose, NZ9000Glc<sup>−</sup>Lac<sup>+</sup> excreted equivalent amounts of glucose (17.1 mM) compared to the lactose consumed (Fig. 3a and b). When grown in a fermentor with the pH controlled at 6.5 and lactose as the limiting factor, *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> grew to about half of the cell density of NZ9000Lac<sup>+</sup> (Fig. 3c and d). *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> produced equimolar amounts of glucose (52.7 mM) from lactose (52.3 mM) under these conditions, while NZ9000Lac<sup>+</sup> metabolized both the galactose and the glucose moiety of lactose (Fig. 3c and d). When grown under limiting carbon conditions, *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> reached a lower cell density than NZ9000Lac<sup>+</sup> because only half of the total carbon supplied (the galactose moiety of lactose) could be

metabolized. A slight accumulation of galactose, most probably resulting from dephosphorylation of galactose-6-phosphate, was observed in *L. lactis* NZ9000Lac<sup>+</sup> grown as standing culture and during growth under pH controlled conditions (Fig. 3a and c). To examine whether the characteristics of the strains in synthetic laboratory media would prevail during milk fermentation, *L. lactis* NZ9000Lac<sup>+</sup> and NZ9000Glc<sup>−</sup>Lac<sup>+</sup> were grown in skim milk, in which lactose (~150 mM, which is ~5%) is the main carbon source. Lactose, galactose, and glucose concentrations in the culture supernatants were measured at different growth-stages using HPLC (Fig. 3e and f). As in synthetic medium, lactose (46 mM) was used to produce glucose (up to a concentration of 38 mM) by *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup>, while in NZ9000Lac<sup>+</sup> the lactose concentration decreased only by 29 mM and no glucose was detected. The final pH reached by milk-grown cultures was 4.2, which is the same as that reached by CDM-grown cultures.

### 3.4. Fate of carbon in the glucose and galactose moieties of lactose

To follow the fate of each sugar moiety of lactose during fermentation, lactose metabolism of *L. lactis* NZ9000Lac<sup>+</sup> and *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> was analyzed in vivo by <sup>13</sup>C-NMR using non-growing cells under controlled conditions of pH, temperature and gas atmosphere (Neves et al., 1999). Lactose labeled either on the glucose moiety ([1-<sup>13</sup>C<sub>Glc</sub>]lactose) or on the galactose moiety ([1-<sup>13</sup>C<sub>Gal</sub>]lactose) was used as a substrate. *L. lactis* NZ9000Lac<sup>+</sup> metabolized both the galactose moiety and the glucose moiety of lactose to lactate (Fig. 4a). Galactose accumulated transiently to low levels and was used upon lactose depletion. *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> only metabolized the galactose moiety of lactose and did not accumulate galactose (Fig. 4b). Strain NZ9000Glc<sup>−</sup>Lac<sup>+</sup> did not metabolize the glucose moiety but, instead, glucose derived from lactose was completely excreted. Consequently, *L. lactis* NZ9000Glc<sup>−</sup>Lac<sup>+</sup> produced half of the amount of lactate compared to NZ9000Lac<sup>+</sup>, from the same amount of lactose. Also, a difference in the pattern of intracellular metabolites was detected. In *L. lactis* NZ9000Lac<sup>+</sup> both tagatose-1,6-bisphosphate and fructose-1,6-bisphosphate accumulated up to 24 mM intracellular concentration, whereas in NZ9000Glc<sup>−</sup>Lac<sup>+</sup> only tagatose-1,6-bisphosphate (up to 31 mM) was detected (data not shown).

## 4. Discussion

We show that removing *glk*, *ptnABCD* and *ptcBA* results in a complete blockage of glucose metabolism in *L. lactis* NZ9000. A strain carrying deletions in these three genetic loci cannot use glucose present extracellularly, nor can it metabolize glucose formed intracellularly by lactose-6-phosphate hydrolysis. Glucose derived from lactose-6-phosphate hydrolysis is expelled into the medium. The

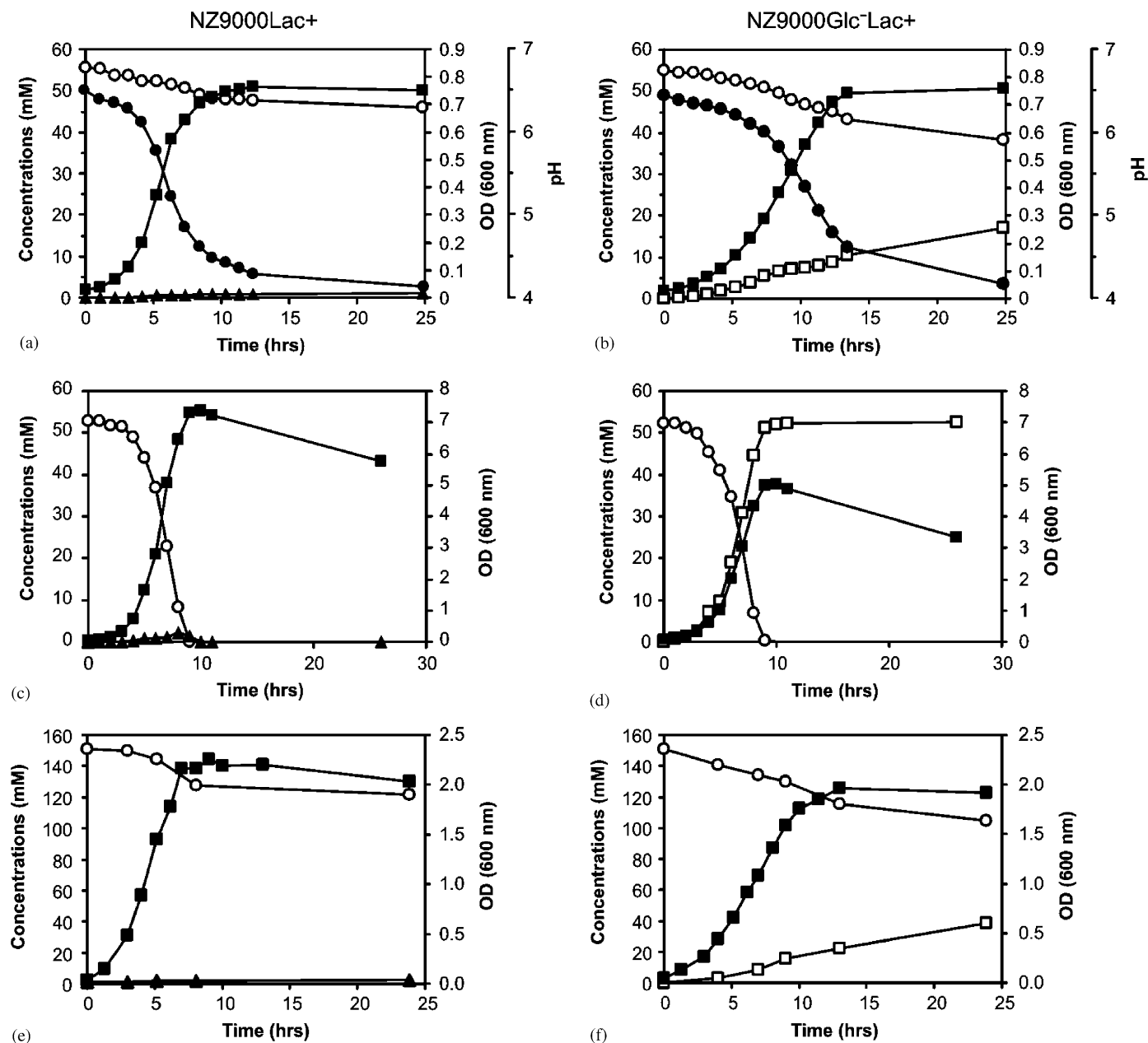


Fig. 3. Growth of *L. lactis* in batch cultures with and without pH control in lactose-CDM and skim milk. Culture-pH and extracellular concentrations of lactose, glucose, and galactose measured during growth of *L. lactis* NZ9000Lac<sup>+</sup> (a, c, e) and *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> (b, d, f) in CDM with 2% lactose (w/v) without pH control (a,b), with the pH controlled at 6.5 in a fermentor (c, d), and grown in skim milk without pH-control (e, f). Symbols: growth OD<sub>600</sub> (■), pH (●), lactose (○), glucose (□), galactose (▲). Data are from a representative experiment. Standard errors (not shown) never exceeded 10% of the given value.

system responsible for glucose excretion has not yet been identified but it is probable that a non-PTS permease is involved, since passive diffusion of glucose over the cell membrane has not been reported. Previously, a glucokinase- and PTS<sup>man/glc</sup>-deficient mutant of *L. lactis* ssp. *lactis* strain 133, which was blocked in glucose metabolism, was obtained by classical mutagenesis (Thompson et al., 1985). Interestingly, we show now by transcriptome analysis and self-cloning techniques that, in addition to a glucokinase and PTS<sup>man/glc</sup> deletion, removal of a PTS<sup>cel</sup> is necessary to completely prevent glucose fermentation, at least in

*L. lactis* ssp. *cremoris* NZ9000. The possibility that *L. lactis* strain 133 does not have a PTS<sup>cel</sup> with the ability to use glucose cannot be disregarded, but this is unlikely since *L. lactis* strains 133 and IL1403 belong to the same subspecies and the latter does contain *ptcBAC*, the genes encoding PTS<sup>cel</sup>. Most probably, the classical mutagenesis approach led to genetic changes that went undetected (Nielsen, 2001).

The decreased maximum growth-rates and higher final cell densities reached by the strains deleted for glucokinase alone or together with a deletion in PTS<sup>man/glc</sup> or PTS<sup>cel</sup> cannot be fully explained at this point. Obviously, deletion



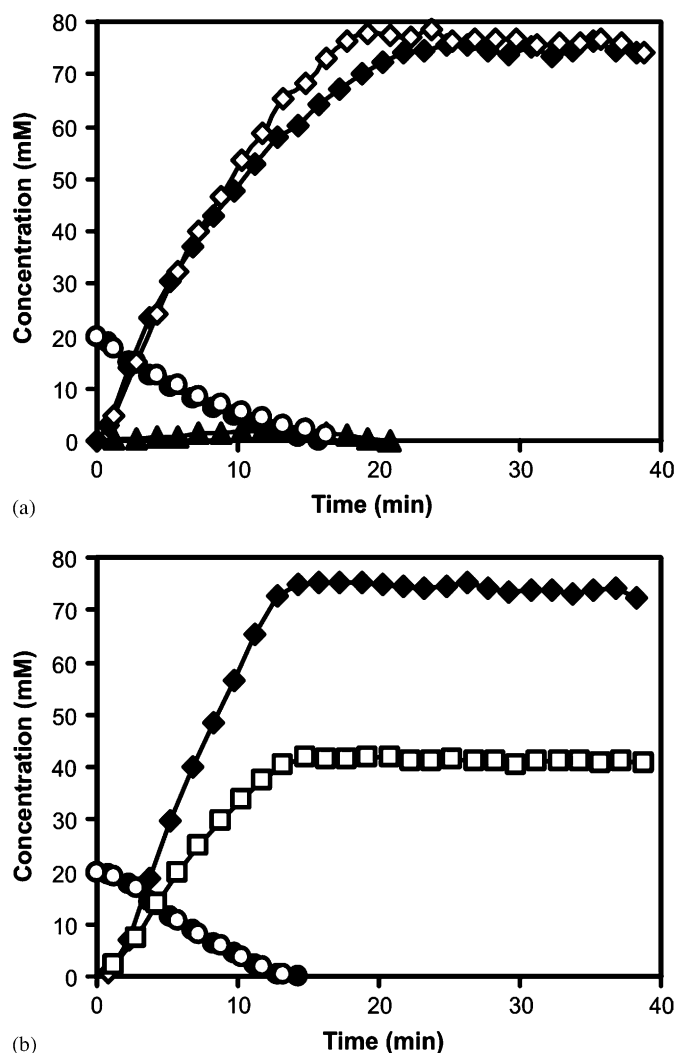


Fig. 4. In vivo analysis of lactose metabolism using NMR. Kinetics of 20 mM  $[1-^{13}\text{C}_{\text{gal}}]\text{lactose}$  and 20 mM  $[1-^{13}\text{C}_{\text{glc}}]\text{lactose}$  consumption and concomitant product formation by non-growing cells of *L. lactis*, performed under anaerobic conditions at 30 °C and at pH 6.5. (a) *L. lactis* NZ9000Lac<sup>+</sup>; (b) *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup>. Symbols:  $[1-^{13}\text{C}_{\text{gal}}]\text{lactose}$  (●),  $[1-^{13}\text{C}_{\text{glc}}]\text{lactose}$  (○), lactate from  $[1-^{13}\text{C}_{\text{gal}}]\text{lactose}$  (◆), lactate from  $[1-^{13}\text{C}_{\text{glc}}]\text{lactose}$  (◇), glucose (□), galactose (▲).

of glucose metabolic genes will decrease the metabolic efficiency and thereby affect the growth-rate. Sugar metabolism is a highly regulated process and disruption of genes involved, especially those encoding PEP-PTS components, might have a big regulatory impact on the overall sugar metabolism. *L. lactis* strains with mutations in glucose transport systems showed a shift to a mixed-acid fermentation (data not shown) and therefore it is likely that under non-controlled conditions of pH, autoacidification is slower allowing these strains to reach a higher biomass than the wildtype, *L. lactis* NZ9000.

Furthermore, we demonstrate that the PTS<sup>man/glc</sup> and the PTS<sup>cel</sup> are the only two phosphotransferase systems able to use glucose as a substrate in *L. lactis* NZ9000. PTS's can be specific for more than one sugar (Postma et al., 1993), although, to our knowledge, a PTS with

specificity for both cellobiose and glucose, such as PtcBAC identified here, has not been reported. In addition to using these two glucose PTS's, *L. lactis* can import glucose via one or more non-PTS permease(s), which remain elusive. Our results show that the DNA-microarray technique is a useful tool for rational screening, e.g., to pursue new metabolic engineering strategies.

Growth of *L. lactis* without pH control, as in natural milk fermentations, is halted when the pH reaches values below 4.2. Under these conditions, *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> uses more lactose than NZ9000Lac<sup>+</sup> before growth ceases, since only the galactose moiety of the available lactose is converted to lactate, with concomitant acidification, leaving the glucose untouched. Therefore, in addition to the production of glucose, the residual level of lactose in the medium is lower than that in the medium of a fully grown culture of NZ9000Lac<sup>+</sup>. Most importantly, *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> also uses more lactose from the medium and produces glucose during fermentation in skim milk, which is a good indication that this strain is suitable for use in milk fermentations.

NZ9000Glc<sup>-</sup>Lac<sup>+</sup> produces glucose from lactose under all conditions tested, as shown in Figs. 3 and 4. Furthermore, in vivo NMR coupled to specifically labeled lactose showed that *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> directly uses galactose-6-phosphate, formed by lactose-6-phosphate hydrolysis for metabolism, while the glucose moiety of lactose-6-phosphate is expelled into the medium. *L. lactis* NZ9000Lac<sup>+</sup> accumulates a transient, low level of galactose, which is consumed upon lactose depletion. This transient extracellular accumulation of galactose indicates that the cell has a clear preference for utilization of the glucose moiety of lactose. Most likely, free galactose results from dephosphorylation of galactose 6-phosphate by a phosphatase as the result of an inducer expulsion mechanism. Such a two-step reaction of inducer expulsion caused by glucose has been described before in *L. lactis* and other Gram-positive bacteria (Reizer et al., 1983; Thompson and Saier, 1981; Ye and Saier, 1995). When lactose is depleted, the extracellular galactose is used via the Leloir pathway (Grossiord et al., 2003).

In summary, *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> is functional in two ways. First, it could be used to produce glucose from lactose, which could serve as a natural sweetener in fermented dairy products. This in situ produced glucose could replace, at least in part, the frequent addition of other sweeteners to dairy products, as the sweetness of glucose is about 60% of that of sucrose (Schiffman et al., 2000). The production of glucose in combination with the lower acidification achieved by *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> might give rise to a milder tasting end-product, e.g., when the strain is used as an adjunct starter culture in cheese or buttermilk production. Second, *L. lactis* NZ9000Glc<sup>-</sup>Lac<sup>+</sup> uses only the galactose moiety of lactose for growth, which leads to more effective lactose removal from the medium. Therefore, the strain could be used as a nutraceutical to produce milk fermentation products with lower residual lactose concentrations, which would be suitable in a diet for individuals suffering from lactose intolerance.

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